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Intramural Methotrexate Therapy for the Prevention of Neointimal Thickening After Balloon Angioplasty

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Objectives. **The study was performed to test the hypothesis that high local, intramural concentrations of antineoplastic agents at the site of balloon injury inhibit vascular smooth muscle cell proliferation without systemic toxicity.**

Background. **The predominant mechanism for recurrent stenosis after coronary balloon angioplasty is neointimal thickening due to medial smooth muscle cell proliferation. The clinical use of potent antiproliferative agents to prevent restenosis has been limited by the potential for severe systemic side effects. Local therapy with these agents may be effective and free of systemic complications.**

Methods. **After bilateral balloon angioplasty of the carotid arteries of 14 juvenile farm pigs, the dilated arterial segments were treated locally with methotrexate (6.25 mg/ml, total dose 25 mg) or 0.9% saline solution through a perforated balloon catheter. The animals were then killed 30 days after balloon injury to determine the effects of this therapy on neointimal thickness. In an additional six animals, tritium-labeled methotrexate was used to determine the concentration and duration of detectability of methotrexate in the wall of the treated arteries and in the systemic circulation.**

Results. **Two hours after drug instillation the concentration of labeled drug was >1,000-fold greater in the wall of the treated artery than in circulating blood, and this ratio remained between 50 and 100 for at least 7 days. Despite this difference, the mean intimal thickness 30 days after the procedure was similar in the 10 methotrexate-treated arteries and the 18 saline-treated arteries (59 ± 30 vs. 56 ± 25 μm, p = 0.6). The morphologic appearance of the neointima was similar in each group and suggested an important role for mural thrombus in the genesis of the intimal thickening.**

Conclusions. **Treatment with intramural methotrexate, delivered through a perforated balloon catheter at the selected concentration and total dose, failed to prevent intimal thickening after balloon injury. Nonetheless, the perforated balloon catheter appears to be a promising means of delivering a high local concentration of drugs with potentially life-threatening systemic side effects. The optimal concentrations and combinations of candidate drug therapies warrant further evaluation.**

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Balloon angioplasty of atheromatous coronary artery stenoses is followed by recurrent stenosis at the site of arterial injury in 35% to 40% of patients (1,2). The predominant cause is formation of a fibrocellular neointima (3–5). The cellular events that characterize the fibroproliferative response to arterial injury have been well described. They include the deposition of platelets on the disrupted endothelial surface, the release of mitogenic factors from platelets and monocytes, the proliferation and migration of smooth muscle cells from the media to the intima and, finally, the synthesis and secretion of connective tissue matrix (6,7). Conventional pharmacologic therapies directed at the inhibition of platelet activation, thrombus formation or vasoconstriction have failed to reduce the incidence of restenosis in clinical studies (8–12). Antiproliferative therapies have been inadequately evaluated in clinical studies, principally because of the possibility of life-threatening systemic side effects. One potential means of avoiding these side effects was recently described by Wolinsky and Thung (13). These investigators used a specially designed perforated balloon catheter to inject fluids directly into the wall of canine brachial arteries. Fluoresceinated heparin, infused through the catheter, was shown to be uniformly distributed throughout the arterial wall for at least 48 to 72 h.

We hypothesized that with the use of the perforated balloon catheter, high intramural concentrations of a potent antiproliferative drug could be achieved at the site of balloon angioplasty and that this local pharmacologic therapy might

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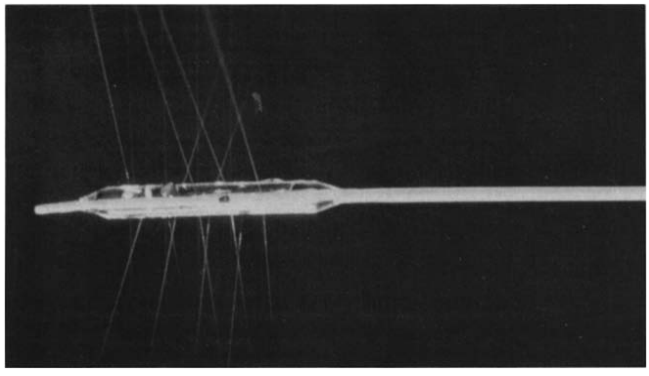


Figure 1. The perforated infusion balloon catheter. Infusate is delivered directly into the arterial wall through a series of 25-μm diameter holes. Reprinted, with permission, from Wolinsky and Thung (13).

prevent medial smooth muscle cell proliferation and restenosis without a significant risk of systemic side effects. Of the antineoplastic drugs commonly used to treat malignant neoplasms of mesenchymal origin, methotrexate was considered the most suitable agent for evaluation. It has potent antiproliferative, anti-inflammatory and antifibrotic activity and has been successfully used to treat of a variety of other nonmalignant conditions including psoriasis, rheumatoid arthritis and hepatic cirrhosis.

The principal aims of this study, therefore, were 1) to determine the duration of detectability, and to estimate the concentration, of methotrexate in the arterial wall after delivery through a perforated balloon catheter, and 2) to determine the effects of local intramural methotrexate therapy on the extent of neointimal thickening after balloon angioplasty of porcine carotid arteries.

Methods

Drug delivery system. The study was performed with the approval of the University of Michigan Committee on the Use and Care of Animals and was conducted in two phases. In the first phase, tritium-labeled methotrexate was used to estimate the concentration of methotrexate at the site of infusion of the drug through the balloon catheter over 14 days. In the second phase, the effect of this therapy on the degree of neointimal thickness was determined 30 days after balloon dilation. The drug delivery system used in the study was the Wolinsky infusion balloon catheter (USCI Division of CR Bard Inc.). The catheter has a 4.3F triple-lumen shaft with a distal balloon made of polyethylene tetraphthalate. The balloon (Fig. 1) has 28 holes (25 μm in diameter, in longitudinal rows) through which the infusate is delivered.

Experimental protocol. The study protocol was similar in the two phases. After sedation with telazol (6 mg/kg body weight) and xylazine (2.2 mg/kg), juvenile Yorkshire farm pigs weighing 18 to 28 kg were intubated and anesthetized with 1% halothane. Access to the carotid arteries was obtained by cutdown through a midline cervical incision. Before direct manipulation, the outer diameter of each artery was measured, and the size of the inner lumen was estimated by using the known approximate wall thickness. A conventional balloon catheter (approximate balloon/artery ratio, 1.5:1; balloon size, 2.75 to 4 mm) was then introduced into each artery under direct vision through a transverse arteriotomy and inflated 1 cm distal to the arteriotomy wound to 10 atm for 60 s. A perforated balloon catheter (balloon diameter 3 to 3.5 mm) was then prepared with either methotrexate for use in the left carotid artery or with saline solution for use in the right carotid artery. Special care was taken to ensure that the infusion catheter was free of air bubbles and that the infusate was delivered directly to the site of balloon injury. A different balloon catheter and inflation device were used on each side to ensure that the balloon pores had not become occluded during the first inflation and that no active drug was infused on the control side. The arteriotomy and skin wounds were then closed and the animals were allowed to recover. Each pig was treated with oral aspirin (150 mg/day) for at least 3 days before the procedure and daily postoperatively. Intravenous heparin (150 to 200 U/kg) was given before balloon inflation, but no further anticoagulant therapy was administered after the procedure. All animals were given a normal laboratory chow diet throughout the study period.

Phase 1. In the first phase, six pigs underwent bilateral carotid balloon angioplasty and infusion of radiolabeled methotrexate. Tritiated methotrexate (New England Nuclear) with a specific activity of 1,000 μCi/ml (0.0093 mg/ml) was diluted with 0.9% saline solution to a concentration of 12.5 μCi/ml. Over 30 to 60 s, a total dose of 40 μCi of labeled methotrexate was infused at a pressure of 5 to 6 atm through the perforated balloon catheter into the left carotid artery at the site of balloon angioplasty. An equivalent volume of 0.9% saline solution was infused into the opposite carotid artery by using the same inflation variables. Each of the six pigs was then killed under general anesthesia with a lethal dose of Pentothal Sodium at a different time interval (2, 24, 48 or 72 h or 7 or 14 days) after instillation of the labeled drug.

Each carotid artery was then resected, and a blood sample was obtained for determination of the circulating tritiated methotrexate activity. The arterial specimens, trimmed to include only the length of the balloon-injured segment, and the blood samples were then weighed and individually oxidized in a sample oxidizer (Packard Instrument Company, model 306). The resulting water vapor was collected, condensed and transferred to a liquid scintillation counter (Packard Instrument Company, model 4530) for determination of the beta-emitting activity of the tritiated water. The activity, expressed as μCi/g of tissue, was determined by using the measured counts/min, the known half-life of tritium, the dose delivered and the elapsed time from instillation to the animal's death.

Phase 2. In the second phase, 10 pigs underwent bilateral carotid balloon angioplasty and infusion of methotrexate

(6.25 mg/ml) into the left carotid artery through a perforated balloon catheter with a balloon diameter of 3 or 3.5 mm. A total of 4 ml (25 mg) was infused at a pressure of 5 to 6 atm over 30 to 60 s. An equivalent volume of normal saline solution was infused over the same period and at the same pressure into the contralateral artery. After completion of the procedure, a blood sample was obtained for determination of the systemic methotrexate level. Because of the possibility that the contralateral untreated artery might take up circulating methotrexate, four additional pigs underwent bilateral angioplasty and arterial infusion of 0.9% saline solution to serve as "true controls." Each of these 14 pigs was then killed 30 days after the angioplasty and infusion procedures.

Histologic analysis. In each phase of the study, the animals were killed under general anesthesia with a lethal dose of intravenous Pentothal Sodium. The carotid arteries were exposed and perfusion fixed in situ with 10% neutral buffered formaldehyde. The balloon-injured arterial segments were then resected, paraffin-embedded, sectioned and stained with hematoxylin-eosin and Movat pentachrome stains. The morphologic features of the treated and nontreated arterial segments were compared by an experienced pathologist (G.D.A) who was unaware of the treatment given to each arterial segment. The extent of neointimal thickening, measured from the internal elastic lamina to the lumen surface at the point of maximal thickening, was determined for each arterial segment by using an ocular micrometer. Intimal thickness was also measured at circumferential points 90°, 180° and 270° from the point of maximal thickening; and the four measurements were averaged to give a mean intimal thickness.

Statistics. All values are recorded as the mean value ± SEM unless otherwise stated. Comparisons of the severity of maximal and mean intimal thicknesses were performed by using the Mann-Whitney *U* and Kruskal-Wallis tests. A two-tailed p value <0.05 was considered to be statistically significant. The sample size was chosen to give a power of 80% to detect a 50% reduction in intimal thickness in the treated group by using an assumed control neointimal thickness of 250 μm.

Results

Duration of methotrexate activity. The activity of tritium-labeled methotrexate in the treated carotid artery at each time interval is shown graphically in Figure 2. Two hours after instillation of the labeled methotrexate, the tissue activity was 10.4 μCi/g (2.14×10^{-4} μmol/g). At the same time interval, the activity in the saline solution-treated contralateral artery was 0.38 μCi/g. The activity in the treated artery decreased over the 1st 24 h to 3.9 μCi/g but remained >2.5 μCi/g at each interval from 24 h to 7 days (Fig. 2). At 14 days, the level was 0.33 μCi/g. In the saline solution-treated contralateral carotid artery, the activity also

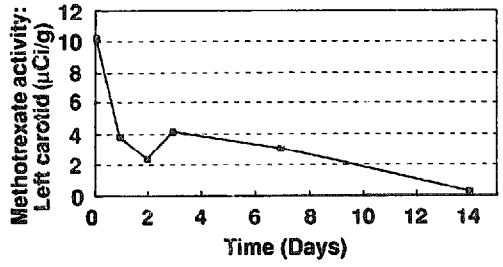


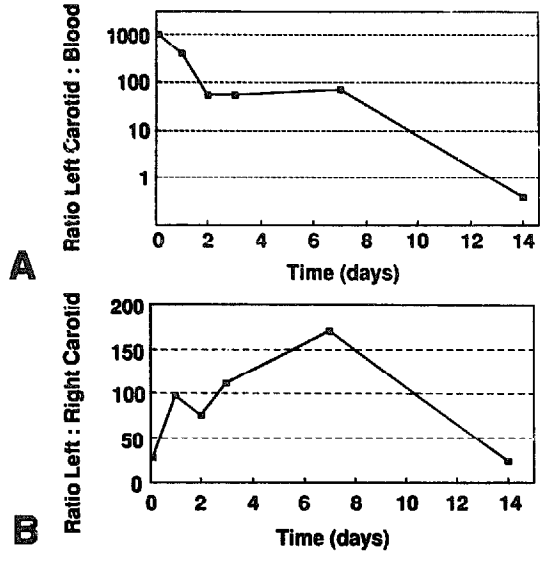
Figure 2. Time-activity curve of tritium-labeled methotrexate (expressed as μCi/g of tissue) in the treated carotid arteries.

decreased over the 1st 24 h and subsequently remained <0.04 μCi/g at each time interval.

The ratio of activity in the treated carotid artery to that in the circulating blood at each time interval is shown in Figure 3A. Two hours after instillation of the labeled methotrexate, the tissue concentration of methotrexate was >1,000 times greater than the blood concentration. The ratio decreased over the 1st 48 h but remained >50:1 for the 1st 7 days. Two weeks after methotrexate instillation, the tissue activity was approximately the same as the activity in the circulating blood (Fig. 3A). Similarly, the tissue activity of labeled methotrexate was considerably greater in the treated than in the nontreated carotid artery for at least 7 days and remained approximately 25 times greater 14 days after drug instillation (Fig. 3B).

Effect on neointimal thickening. Ten pigs were treated unilaterally with methotrexate in the second phase of the study and were allowed to survive for 30 ± 1 days postoperatively. In these animals, the serum methotrexate level, measured 1 h after completion of the drug instillation, was 1.15 ± 0.31 μmol/liter. Because of the nature of the study

Figure 3. A, Ratio of the activity of labeled methotrexate in the treated left carotid artery and in the circulating blood over the 2-week study period. B, Ratio of the activity of labeled methotrexate in the treated left and untreated right carotid arteries.



protocol, absolute tissue concentrations of methotrexate in the treated carotid arteries could not be directly measured. However, by using the tissue/blood ratio derived in the first phase of the study, it can be estimated that the carotid tissue concentration of methotrexate immediately after drug infusion was approximately 1,000 μmol/liter and remained approximately 100 μmol/liter for at least 7 days. Each of the treated pigs remained healthy throughout the study period. Blood was not drawn for regular hematologic and biochemic analysis, but no animal had any overt side effects from the methotrexate therapy.

Histologic examination of the resected carotid arteries 30 days after balloon injury showed a spectrum of morphologic appearances of the neointima. Several arteries showed minimal evidence of previous injury. In these vessels (two methotrexate-treated, two saline-treated and one "true control"), the media, internal elastic lamina and intima showed only very minor changes, suggesting that the balloon injury had been inadequate to induce an intimal proliferative response. In contrast, two arteries (one methotrexate-treated and one control) were considerably narrowed by large, eccentric lesions that had the histologic appearance of organizing mural thrombus with residual fibrin, hemosiderin-laden macrophages, smooth muscle cell infiltration and new capillary formation; one additional animal, killed on day 7 in phase 1 of the study, was noted to have complete thrombotic occlusion of one arterial segment.

The remaining 21 arteries showed features more typical of fibrocellular neointimal thickening. In these vessels, the proliferative response was commonly eccentric and was usually maximal in proximity to a region of apparent disruption of the internal elastic lamina and adjacent media (14). In one artery (Fig. 4A), the internal and external elastic laminae were disrupted with features suggestive of trauma induced by a high pressure jet from the perforations of the balloon catheter. In the remaining arteries, only partial thickness mural damage was apparent. The neointima appeared to consist of a heterogeneous population of cells. Although immunohistochemical stains were not used to confirm cell types, the appearances frequently suggested not only proliferation of smooth muscle cells but also heavy infiltration of the lesion by macrophages, often laden with hemosiderin, occurring singly or as foreign body giant cells. The presence of the latter cells suggests that foreign particulate matter may have been introduced into the arterial wall from the balloon catheter. Calcification, observed in two vessels, occurred in both the media and the neointima (Fig. 4B).

The mean intimal thickness was determined in each artery by using measurements at the point of maximal thickness and in the remaining three quadrants. The maximal thickness was 195 ± 112 μm in the methotrexate-treated group compared with 148 ± 74 μm in the saline-treated arteries ($p = 0.6$); the mean intimal thicknesses were 59 ± 30 μm and 56 ± 25 μm, respectively ($p = 0.16$) (Fig. 5). There were no significant differences in maximal or mean intimal thickness between the "true control" and group and the group with infusion of saline solution into the contralateral artery. Similarly, no difference between the groups was apparent if the pigs with atypical histologic findings (minimal injury or organizing thrombus) were excluded from the analyses.

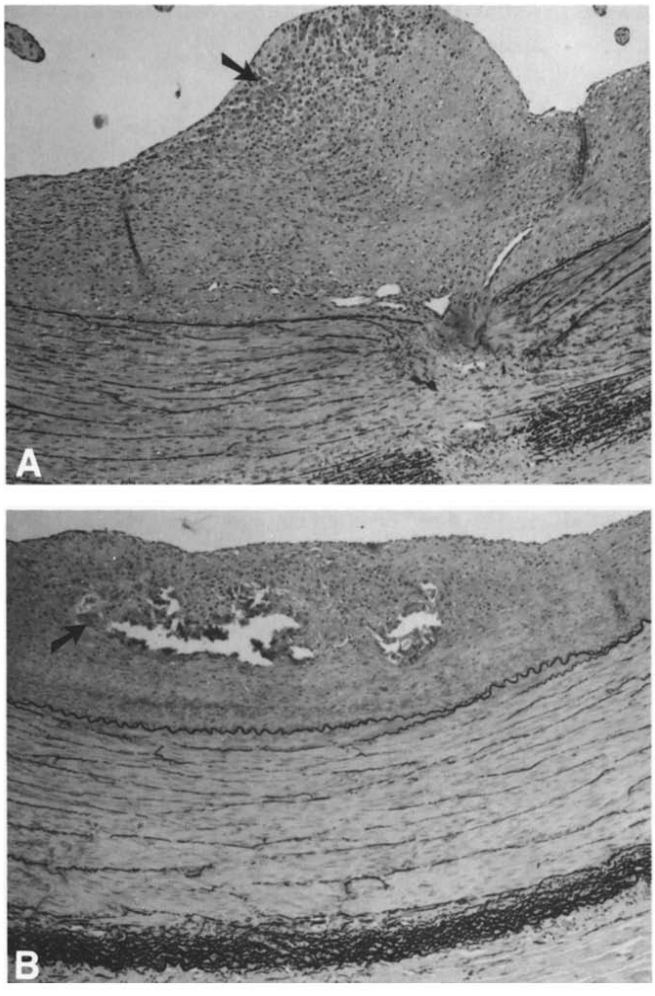


Figure 4. A, Eccentric intimal thickening in close proximity to a breach in both the internal and external elastic laminae in a methotrexate-treated artery. The lesion is composed predominantly of smooth muscle cells and connective tissue matrix but also has a moderately heavy infiltration of hemosiderin-laden macrophages (arrow) adjacent to the lumen surface (Movat stain). Reprinted, with permission, from Muller et al. (14). **B,** The contralateral saline-treated artery of the same animal. A similar degree of intimal thickening is present. The neointima contains foci of calcification with foreign body giant cells (arrow). The underlying internal elastic lamina and media appear relatively normal (Movat stain).

Discussion

Failure of antiproliferative therapy to prevent neointimal thickening. The principal findings of this study were that, with the use of the perforated balloon catheter, high tissue concentrations of methotrexate were achieved in the arterial wall at the site of balloon injury and remained present for at least 7 days. Despite this treatment, there was no difference

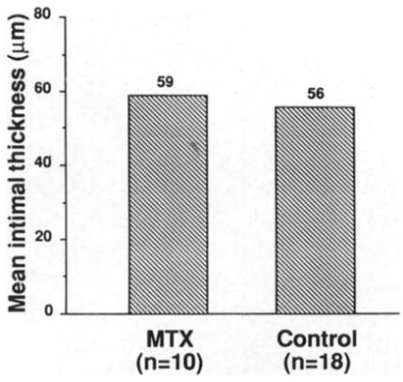


Figure 5. Mean intimal thickness (μm) in the methotrexate-treated (MTX), and saline-treated (control) carotid arteries. Differences between the two groups were not statistically significant.

in the degree of the neointimal thickening in the treated and nontreated carotid arteries 30 days after balloon injury. The morphologic appearance of the neointima, although somewhat heterogeneous, was also similar in each of the groups; there was no evidence of an adverse effect of the methotrexate therapy. It is possible, however, that the perforated balloon catheter may have contributed to the degree of arterial injury. In one pig, the morphologic features suggested that the high pressure jets of the balloon catheter had caused transmural arterial injury with disruption of both the internal and the external elastic laminae. Several potential mechanisms may explain the apparent failure of local antiproliferative therapy to prevent trauma-induced neointimal thickening. These mechanisms include an inadequate tissue concentration of methotrexate, nonuniform distribution of drug within the arterial wall, an inadequate duration of antiproliferative therapy, thickening of the neointima by infiltration of nonproliferating cells or thrombus and the detrimental effects of inhibition of endothelial cell regeneration.

Concentration and duration of therapy. The findings of the first phase of the study suggest that high concentrations of methotrexate were present in the arterial wall for at least 7 days. The optimal therapeutic blood concentrations of methotrexate in malignant neoplasms for which methotrexate is commonly used are listed in Table 1 (15). The estimated intramural methotrexate concentration of 100 to 1,000 μmol/liter is comparable to the order of magnitude of the peak serum methotrexate concentrations recommended for the treatment of the least responsive malignant neoplasms, such as large cell adenocarcinoma and osteosarcoma. Thus, the local concentrations of methotrexate should have been adequate to achieve an antiproliferative effect for at least 1 week. Whether the drug was uniformly distributed within the arterial wall is less clear. Although Wolinsky and Thung (13) showed a uniform distribution of fluoresceinated heparin and horseradish peroxidase throughout the full thickness of the canine arterial walls after delivery through the same perforated balloon catheter, the distribution of methotrexate within porcine arteries in this study is unknown. However, the drug has a small molecular weight (450 daltons), allowing it to diffuse readily, and is actively taken up across the cell membrane. It is not unreasonable to assume, therefore, that the distribution of the drug would have been similar to that shown with other agents in previous studies.

Table 1. Optimal Therapeutic Peak Serum Concentrations of Methotrexate (15)

Malignant Neoplasm	Methotrexate Concentration (μmol/liter)
Osteosarcoma	1,000
Large cell adenocarcinoma	100
Lymphoma	10
Choriocarcinoma	1

What time interval constitutes an adequate duration of therapy in this model of restenosis is also unknown. It is known from careful studies performed by Clowes et al. (16) that medial smooth muscle cell division begins between 24 and 72 h after balloon denudation of rat carotid arteries and is followed soon by migration of the proliferating cells from the media to the intima. In the porcine carotid artery, medial smooth muscle cell proliferation is well advanced within 48 h of balloon injury (17); intimal thickening is apparent within 7 days and peaks at approximately 28 days (18). In this study, high local concentrations of antiproliferative therapy were present for 7 to 14 days. It is possible that therapy for the full duration of the study period, for example by retreatment with methotrexate at weekly intervals, may have been more effective in preventing neointimal thickening. The findings of this study are consistent, however, with those of Murphy et al. (19). In their study, which was also performed in a porcine model, methotrexate (1.25 mg 5 days/week orally or 20 mg/week intramuscularly) and azathioprine (25 mg/day orally), given throughout a 28-day study period, failed to inhibit neointimal thickening in coronary arteries after implantation of oversized metallic stents.

Composition of the neointima. A substantial proportion of the cells contributing to the thickness of the neointima may have been nonproliferating cells. In one study (20), approximately 50% of the smooth muscle cells that migrated from the media to the intima of balloon-injured rat carotid arteries were nondividing cells; and in this study, the lesions produced frequently had a heavy infiltration of cells with the morphologic appearances of macrophages. Mural thrombus appears to have played an important role in the genesis of the intimal thickening. Thrombus is known to stimulate smooth muscle cell proliferation directly (21,22), and histologic examination of material obtained by directional coronary atherectomy (23) suggests that incorporation of mural thrombus plays a much greater role in the development of human restenotic lesions than has previously been appreciated. These findings suggest that restenosis is a heterogeneous response to injury and that in addition to antiproliferative

therapy, concomitant potent antithrombotic therapy may be necessary to adequately inhibit the restenotic process.

Inhibition of endothelial cell regeneration. One additional explanation for the apparent lack of efficacy of local methotrexate therapy may be that any beneficial effect of the drug on smooth muscle cell proliferation may have been offset by the negative impact of inhibition of endothelial cell regeneration. Studies in the balloon-injured rat carotid artery model have previously shown that restoration of the endothelial covering inhibits medial smooth muscle cell proliferation and that prolonged endothelial denudation is associated with an increased degree of neointimal thickening (16,24). Consistent with this hypothesis are prior observations (25) that methotrexate inhibits endothelial cell proliferation in cell culture to a significantly greater degree than it inhibits fibroblast cell proliferation.

Reliability of the animal model. Several animal models of human restenosis have been used to evaluate the efficacy of therapies for the prevention of intimal proliferation (14,16,18,26–29). Some investigators (18) have used large balloons to cause deep arterial injury in porcine carotid arteries and have correlated the degree of injury with the extent of mural platelet deposition and thrombus formation at the injured site. The apparent relation between the severity of the proliferative response and the presence of focal disruption of the internal elastic lamina has been noted previously, both in experimental studies (30,31) and in human postmortem studies (32–34). A potentially more reliable means of inflicting injury on the vessel wall and of increasing the degree of intimal proliferation was recently described by Schwartz et al. (30,31). These investigators used oversized metallic stents to produce intimal proliferation in porcine coronary arteries and showed a close relation between the extent of intimal thickening and the depth of penetration in the arterial wall of the metallic wires. By reducing the variability of the response and by allowing correction of the degree of thickening for the measurable degree of injury, this model may therefore be more sensitive for the evaluation of pharmacologic therapies.

Limitations. The principal limitations of this study are first, the relatively small number of pigs studied and the somewhat variable arterial response to injury. These factors limit the power of the study to detect small but potentially meaningful therapeutic effects. Second, only one drug dose was evaluated. The dose chosen was determined by the maximal concentration of commercially available methotrexate. More concentrated solutions of methotrexate might have been more effective. Preliminary testing using tritium-labeled methotrexate suggested, however, that the dose and concentration used were adequate to achieve very high local methotrexate levels; and for this reason, no higher concentration was tested. Third, because the size of the carotid arteries was not measured directly or estimated angiographically, it is possible that the perforated balloon catheter may have been undersized in some pigs (leading to loss of methotrexate into the systemic circulation) or oversized in others, leading to further carotid artery injury. Although the latter is possible, manipulation and balloon angioplasty of the carotid artery in each pig resulted in vasoconstriction at the site of dilation and in the adjacent arterial segments. This ensured that the subsequently placed perforated balloon catheter was in apposition to the arterial wall over its entire length during drug or saline instillation. Finally, the model selected used nondiseased porcine arteries to create a lipid-free, smooth muscle cell proliferative lesion; whether these results apply equally to atherosclerotic arteries is unknown.

Conclusions. In the balloon-injured porcine carotid artery model of human restenosis, high concentration local intramural methotrexate therapy failed to prevent neointimal thickening. The histologic appearances noted in this study suggest that effective inhibition of neointimal thickening and restenosis after balloon angioplasty may require a combination of potent antiproliferative, antithrombotic and antifibrotic therapies. The perforated balloon catheter represents one promising means for delivering such drugs in high concentrations directly to the site of injury and of maintaining high local tissue concentrations for at least 7 days without the risks of life-threatening systemic side effects.

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